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Systemic Signaling and Local Sensing of Phosphate in Common Bean: Cross-Talk between Photosynthate and MicroRNA399

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ABSTRACT Shoot-to-root communication is crucial for plant acclimation to phosphorus (P)-deficiency. Both sugars and miRNAs have been implicated as potential signal molecules transported through phloem from shoot to root for the regulation of gene expression and Pi uptake in the root. By studying the expression patterns of both a serine/threonine phosphatase gene (*PvHAD1*) and microRNA399 (miR399) in common bean (*Phaseolus vulgaris* L.), we provide evidence for the interaction between light, phloem transport, and miR399 in the systemic regulation of gene expression under P-deficiency. Especially, miR399 expression in both the shoot and the root requires photosynthetic carbon assimilation during the onset of P-deficiency. In contrast to systemic signaling, local sensing was the primary causal factor for rapid down-regulation of *PvHAD1* by Pi prior to the reduction of miR399 level in P-deficient roots. Furthermore, this initial response to Pi in P-deficient root was also mimicked by the Pi analog, phosphonate (Phi). Our current findings suggest that plants have developed a highly coordinated dual regulatory pathway, namely long-distance signaling of P-deficiency from shoot to root versus local sensing of Pi in the root.

Key words: Roots; nutrient stress; photosynthate; signaling.

INTRODUCTION

While the primary source for carbon (C) in higher plants is from photosynthetic carbon assimilation in the shoot, the uptake of essential mineral macronutrients, such as phosphorus (P), is dependent on the root. For optimal growth under P-deficient conditions, plants exhibit several highly coordinated processes, including: increased root and root hair development, regulated expression of transporter genes, release of organic acids and phosphatase enzymes into the rhizosphere (Vance et al., 2003). The assimilation and subsequent partitioning of carbon between shoot and root are influenced by P-deficiency. The acclimation to P-deficiency is marked in part by the sensing of nutrient status in the shoot and is dependent on photosynthesis and the downward transport of sugars in the phloem from shoot to root. Many P-deficiency-induced genes require sugars for maximal expression (Liu et al., 2005; Karthikeyan et al., 2007; Hammond and White, 2008).

In addition to sugars, another crucial player in P-deficiency response is micro-RNA (miRNA). The identification of miRNA399 (miR399) in *Arabidopsis* as a regulatory molecule has been especially informative towards unraveling the mechanism of systemic regulation of P-deficiency response. MiR399 plays an important role in phosphate (Pi) homeostasis in *Arabidopsis* and rice (Fujii et al., 2005; Sunkar et al., 2005; Chiou et al., 2006; Liu et al., 2009). Like other miRNAs, miR399 regulates gene expression post-transcriptionally and such a direct regulatory relationship is based on sequence complementarity between miR399 and its target mRNA sequences. The presence of miR399 in response to P-deficiency has been confirmed in various plant species, including rice, tobacco, and common bean (Sunkar et al., 2005; Lin et al., 2008; Valdés-López et al., 2008). In particular, miR399 is also detected in phloem sap of pumpkin and oil rapeseed (Pant et al., 2008; Buhtz et al., 2008). MiR399 is induced by P-deficiency in both shoot and root. In addition, miR399 is transported from shoot to root in grafting experiments with *Arabidopsis* and tobacco, and movement of miR399 from shoot to root seems to be important for the onset

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of P-deficiency (Lin et al., 2008). Recently, Liu et al. (2009) proposed a model in which miR399 and sugars are potential signal molecules transported via phloem from the shoot to the root. From the above-mentioned reports, it seems evident that phloem-transported potential signal molecules, namely miR399 and sugars, play a crucial role in the systemic signaling of P-deficiency. Given the highly coordinated nature of plant response to P-deficiency, it is probable that there is an interaction between sugars and miRNAs in regulating gene expression under P-deficiency. Whether miR399 synthesis requires sugars is not known.

The expression of miR399 appears to be partly regulated by a transcriptional activator PHR1, because miR399 expression is reduced in *phr1* knockout mutant (Bari et al., 2006). PHR1 is under posttranslational regulation by SUMO E3 ligase SIZ1 (Miura et al., 2005). Computational analyses of the *Arabidopsis* genome sequence revealed that putative PHR1 binding sites (P1BS) are present in 68% of P-deficiency-induced genes (Misson et al., 2005). However, it is noteworthy that the presence of such consensus sequence does not validate function, unless supported by experimental data (Schünmann et al., 2004). Furthermore, it is also obvious that the promoters of many P-deficiency-induced genes do not possess P1BS sites and are still responsive to Pi fluctuation, indicating that there is a PHR1-independent regulatory mechanism involved in the P-deficiency response (Hammond et al., 2004).

Compared to the extensive documentation of systemic signaling of P-deficiency in plants grown for an extensive period under low Pi, studies are lacking on the mechanism of local Pi sensing in the root. An *Arabidopsis* mutant, *pdr*2, displays altered root architecture due to the loss of local Pi sensing to low Pi. Intriguingly, P-deficiency-induced expression is not impaired in *phr2* compared to wild-type plants (Ticconi et al., 2004). Another *Arabidopsis* mutant, *LPR1*, senses low Pi at root tip and has a short root phenotype (Svistoonoff et al., 2007). It remains unclear whether and/or how the long-distance shoot-to-root signaling interacts with local sensing of Pi for the regulation of gene expression in the root. It is important to determine whether crucial regulatory components in systemic signaling of P-deficiency, such as miR399 and PHR1, also play a role in the local sensing of Pi by the root.

We postulate that plants have acquired a highly sensitive mechanism in the root to directly sense the fluctuation of Pi levels in the rhizosphere, because throughout evolution, plants were subjected to constant Pi-limiting growth conditions of varying severity and Pi-sufficiency seldom occurred. Even if Pi is present as a nutrient patch in soil layers, it is in localized regions of the root system (Svistoonoff et al., 2007). The plasticity of root architecture formation is another indication for a root-autonomous response to nutrient fluctuation in nature (Forde, 2002).

We previously reported that both an extended dark period (24 h) and stem-girdling reduced the expression of P-deficiency-induced genes in the cluster root of white lupin. In the current studies, we combined dark treatment or stem-girdling with Pi supply in common bean (*Phaseolus vulgaris* L.) because it is highly sensitive to both P-deficiency and supply of Pi to P-deficient roots. A phosphatase gene, designated *PvHAD1* (for *P*haseolus *v*ulgaris haloacid dehalogenase), was identified and utilized as a rapid 'indicator' gene. *PvHAD1* expression was very sensitive to Pi supply. Both P-deficient plants and P-sufficient plants were subjected to dark treatment or stem-girdling followed by the reversal of Pi supply, respectively. Under these experimental conditions, we attempted to explore the following two questions: (1) How do P-deficient plants respond to Pi in the root prior to the change of P status in the shoot? (2) Conversely, how do P-sufficient plants respond to the withdrawal of Pi for the onset of P-deficiency?

RESULTS

Protein Structure and Phosphatase Activity

PvHAD1 encodes a polypeptide of 271 amino acid residues with a predicted molecular weight of 30.737 kD and isoelectric point (pI) of 5.55 (Figure 1A). All the three motifs characteristic of the HAD superfamily of phosphatase are present in PvHAD1 (Stewart et al., 2003). In particular, the amino acid residues essential for substrate specificity are also conserved. Despite the overall homology between HAD gene family members, phylogenetic

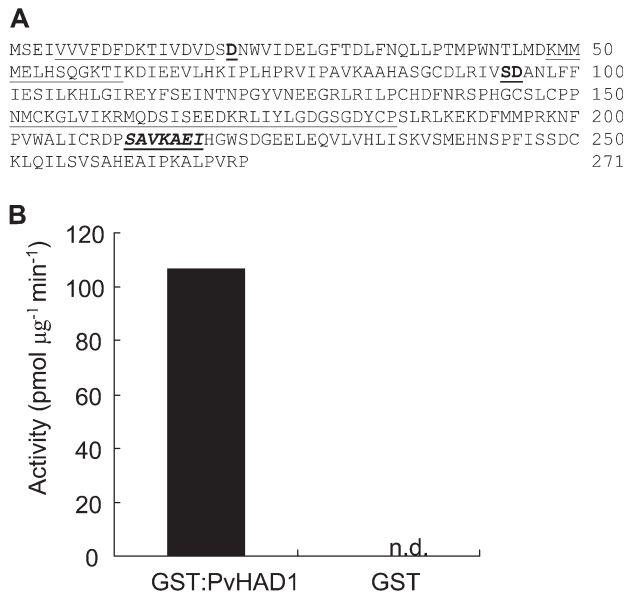

Figure 1. Amino Acid Sequence of PvHAD1 and Serine/Threonine Protein Phosphatase Activity of the Recombinant Protein.

(A) Amino acid sequence of PvHAD1. The three conserved HAD motifs are underlined. Key amino acid residues for substrate specificity are in bold. Putative SUMO domain of seven amino acid residues is underlined and in bold.

(B) *In vitro* serine/threonine protein phosphatase assay. Protein phosphatase activity was detected from purified GST:PvHAD11 fusion protein with phosphopeptide as substrate, but not from free GST as control. Fifty nanograms of fusion protein or GST was used per reaction and enzymatic activity was calculated from reactions for a duration of 10 min at 37 ^{0}C.

analysis revealed that PvHAD1 and homologs from several other plant species such as tomato belong to distinct clades (Supplemental Figure 1A and 1B). In addition, PvHAD1 contains a consensus sequence for SUMO site of seven amino acid (211~217 aa) residues proximal to the C-terminal region. We expressed recombinant PvHAD1 in *E. coli*, purified the GST:PvHAD1 fusion protein, and measured phosphatase activity. The PvHAD1 enzyme displayed high activity using a Ser/Thr phosphopeptide (RRApTVA) (Figure 1B). No activity was detected with free GST. Neither was there activity when p-nitrophenyl phosphate was used as a substrate (data not shown).

Expression of *PvHAD1* under P-Deficiency and Re-Supply of Pi

PvHAD1 expression was induced in the roots of P-deficient bean seedlings 1 week after emergence, and the expression level further increased in P-deficient 2 and 3-week-old plants. While the *PvHAD1* expression level peaked after 3 weeks under P-deficiency, expression in the P-sufficient control plants was non-detectable (Figure 2). Although P-deficiency-induced gene expression is typically reversed by the supply of Pi, the timing of this reversal varies substantially. When we supplied Pi for 2–24 h, *PvHAD1* expression was dramatically reduced within 2 h (Figure 2B). To further monitor how sensitive P-deficiency-induced *PvHAD1* expression is to Pi, we focused on the immediate early response from 15 min to 1 h after Pi supply. Although the *PvHAD1* expression level remained relatively constant for about 30 min, there was a substantial decrease in expression level within 1 h (Figure 2C).

PvHAD1 Expression under N or C Limitation

Balanced application of N and P fertilizers is essential for crop cultivation. Gene regulation under N-limiting conditions is tightly coupled to C status. To test whether N and P, the two most abundant macronutrients, co-regulate gene expression in nutrient-limiting conditions, we probed *PvHAD1* expression in bean plants under various N-starved conditions. *PvHAD1* was not induced by N limitation or depletion for as long as 3 weeks (Figure 3A). Since we observed high sensitivity of common bean to P-starvation, we considered the P status of 3-week-old plants as indicative of an advanced P-deficiency. In a P-deficiency-tolerant plant species, white lupin (*Lupinus albus*), P-deficiency-induced gene expression in cluster roots was dramatically reduced by a 24-h dark period or 24 h after stem-girdling to block phloem transport (Liu et al., 2005). We performed a similar experiment with common bean; neither 24-h dark treatment nor stem-girdling greatly impacted *PvHAD1* expression in roots of 3-week-old P-deficient plants (Figure 3B and 3C).

Regulation of *PvHAD1* by Pi and Phosphonate (Phi) in a Split-Root System

While a traditional split-root experiment is typically performed for the long term (for days or even weeks) in order to demonstrate that both –P and +P halves of the root are under systemic

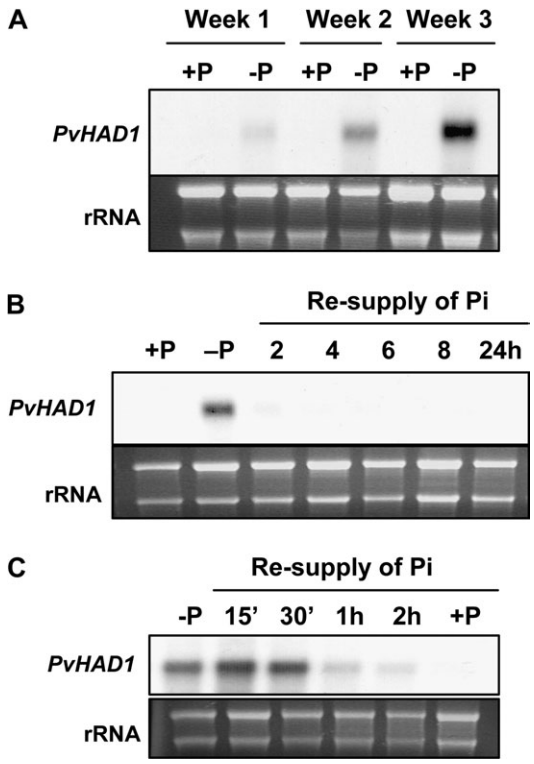

Figure 2. Transcript Abundance of *PvHAD1* in P-Deficient Bean Roots Is Responsive to Pi Supply.

(A) Temporal expression patterns of *PvHAD1* from one to three weeks after emergence of seedlings under +P (1mM Pi) or –P condition (no Pi in nutrient solution).

(B) *PvHAD1* transcripts in P-deficient roots are reduced by the supply of Pi for 2 to 24 h.

(C) *PvHAD1* transcripts in P-deficient roots are reduced within 1 h of Pi supply. RNA blot analyses were performed with total RNA from roots and blots were hybridized with P^{32}-random-labeled *PvHAD1* fragment of full-length coding region. Equal loading was indicated by ethidium bromide staining of rRNA.

regulation by shoot P status, we modified the split-root system to a much shorter period of time to verify local response to Pi. Since the change of P status in the shoot generally takes longer than 1 h (Clarkson and Scattergood, 1982; Dong et al., 1998), we thought that the rapid down-regulation of *PvHAD1* by Pi probably occurred as a root-autonomous response, namely prior to the change in shoot P status from P-deficiency to P-sufficiency. To further evaluate whether this initial response to Pi is a local and specific response in the root, we designed split-root experiments. One half of the root system of a P-deficient plant was subjected to +Pi for 1 h while the other half remained P-deficient, in order to detect any difference of expression between two halves of the root. Phosphonate (Phi), a Pi analog, was also used to mimic Pi and assess local effect of Pi sensing, because plants can neither metabolize nor utilize Phi as nutrient. However, Phi can be sensed by plants as Pi mimic (Carswell et al., 1996). In the control experiments, both halves of the roots of P-deficient plants showed comparable levels of *PvHAD1* expression—an indication that the root

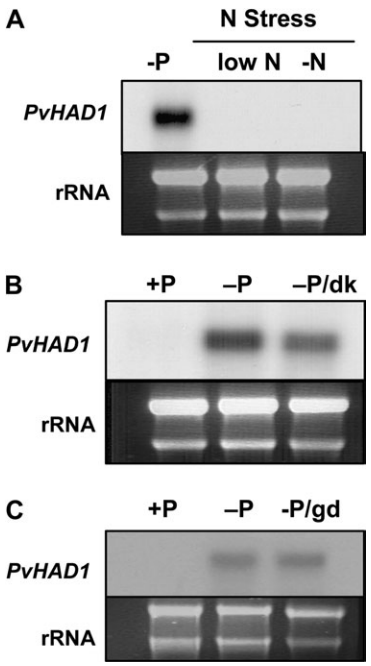

Figure 3. RNA Blot Analyses of *PvHAD1* Expression in N-Deficient or P-Deficient Roots of Dark-Treated or Stem-Girdled Plants.

(A) *PvHAD1* transcripts are not induced by N-deficiency (low nitrate with 0.1 mM or no nitrate in nutrient solution).

(B) *PvHAD1* expression in roots of three-week-old P-deficient plants is not markedly reduced by 24 h dark period (-P/24 h).

(C) *PvHAD1* expression in roots of three-week-old P-deficient plants is not markedly down regulated 24 h after stem girdling (-P/gd). Controls are three-week-old P-sufficient (+P) or P-deficient plants (-P), respectively. Total RNA from roots was separated on denaturing gel, transferred to nylon membrane, and RNA blot was hybridized with random-labeled *PvHAD1* cDNA probe.

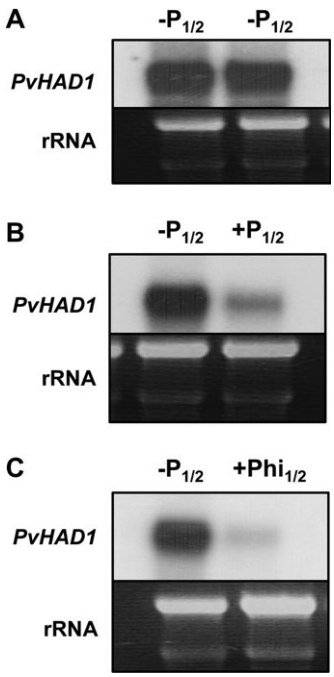

Figure 4. RNA Blot Analyses of *PvHAD1* Expression in a Split-Root System.

(A) In control three-week-old P-deficient plants, each half of the root was maintained in –P nutrient solution for 1 h and *PvHAD1* expression showed no difference between two halves of the root.

(B) *PvHAD1* expression was down regulated in the +Pi half of the root.

(C) *PvHAD1* expression was down regulated in the +Phi half of the root.

Three-week-old P-deficient plants were used for all split-root experiments. The duration of adding Pi or Phi to the split-root is 1 h. Roots were then collected and quickly frozen in liquid nitrogen for RNA extraction. RNA blot analyses were performed with random labeled *PvHAD1* probe. Ethidium bromide staining of rRNA was shown for equal loading of total RNA.

handling during split-root process did not cause any fluctuation of expression. In contrast, the expression level of *PvHAD1* was greatly reduced in the one half of the root treated with +Pi and also in +Phi half of the root (Figure 4). This localized response was quite sensitive to Pi/Phi concentration, because we observed a similar decrease in *PvHAD1* expression at a concentration ranging from 0.25 to 1 mM (data not shown).

Rapid Down-Regulation of *PvHAD1* in P-Deficient Root Is Not Affected by C-Limitation and Is Prior to miRNA399 Reduction

MiR399 plays a crucial role in Pi homeostasis in P-deficient plants under P-starvation. Bari et al. (2006) reported that the miR399 expression in P-deficient *Arabidopsis* is not affected by low-carbohydrate condition. Although miR399 is considered crucial in the P-deficiency signaling cascade, it remains unknown whether the miR399 level correlates with local Pi sensing in P-deficient plants after long-term P-starvation. While our data from the split-root experiments support that the rapid down-regulation of *PvHAD1* in roots is due to the root sensing of Pi, we thought it would be informative to know whether factors in systemic signaling such as C-limitation and miRNA399 interact with local Pi sensing. Three-week-old P-deficient plants were first subjected to 24-h dark treatment or stem-girdling, and then supplied with Pi for 1 h. *PvHAD1* expression in roots of both girdled and dark-treated plants was still down-regulated by 1-h Pi supply (Figure 5A). In contrast, miR399 levels in both roots and shoots were high and unaffected by re-supply of Pi (Figure 5B).

Both *PvHAD1* and miR399 Induction Are Sensitive to C-Limitation at the Onset of P-Deficiency

Rapid down-regulation of *PvHAD1* by Pi in P-deficient roots demonstrates the localized sensitivity of Pi signaling. In efforts to understand Pi sensing and gene expression during the onset of P-deficiency in whole plants, we subjected P-sufficient plants to P-deficient conditions. Recent reports showed that miR399 is induced in the root 24 h after Pi withdrawal and miR399 expression marks the onset of P-deficiency in *Arabidopsis* (Bari et al., 2006; Lin et al., 2008; Liu et al., 2009). Upon abrupt withdrawal of Pi from P-sufficient plants, it is unclear how plants sense the change in Pi availability and initiate P-deficiency response.

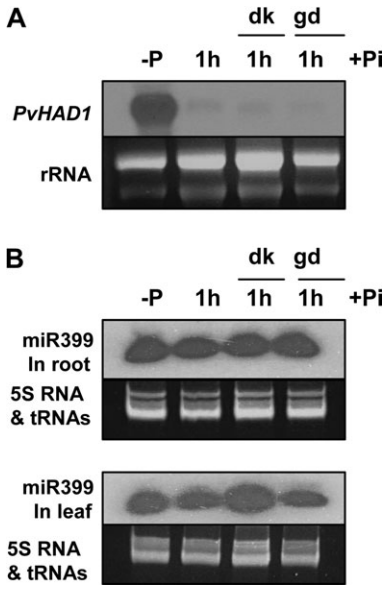

Figure 5. Rapid Reduction in *PvHAD1* Expression by the Supply of Pi Occurs Prior to Any Reduction of miR399 in P-Deficient Plants.

(A) Root *PvHAD1* expression is high in three-week-old P-deficient plants (-P). After 1 h of Pi supply (+Pi) there is dramatic reduction of *PvHAD1* expression level. Similar reduction in *PvHAD1* transcript was also observed if 1 h Pi supply was preceded by 24 h dark treatment (dk) or 24 h after stem girdling (gd).

(B) miR399 expression remains high and constant in both roots and leaves 1 h after Pi supply. Equal loading was indicated by ethidium bromide staining of 5S RNA and tRNAs. End-labeled oligo 399 (reverse complementary to miR399 in *Arabidopsis*) was used as probe to hybridize to RNA blot prepared from denaturing 8 M/polyacrylamide gel. Ethidium bromide staining of rRNA was shown for equal loading of total RNA. Twenty micrograms of total RNA was separated and transferred to Hybond N^+ membrane. Blot was hybridized with random-labeled *PvHAD1* probe.

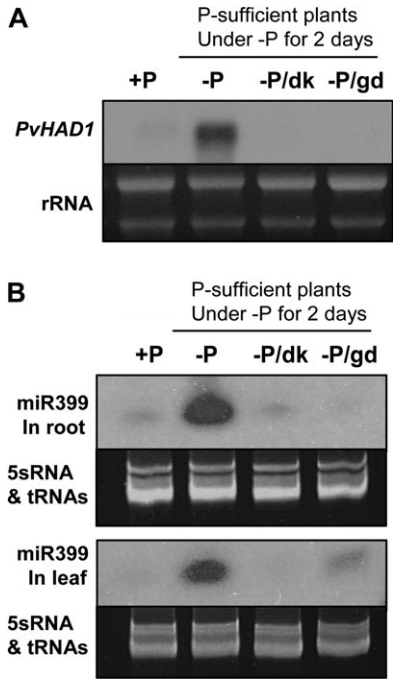

Figure 6. *PvHAD1* and miR399 Expression During the Onset of P-Deficiency.

(A) *PvHAD1* expression in root was induced 2 days after Pi withdrawal (-P) from P-sufficient plants grown under standard 16 h/8 h light/dark photoperiod, but *PvHAD1* was not induced by 2 day Pi withdrawal in plants in continuous dark (-P/dk) or in stem-girdled plants (-P/gd).

(B) miR399 expression in roots and leaves was strongly induced by 2 day Pi withdrawal (-P) in plants under standard light conditions, but not induced in plants in continuous dark or in stem-girdled plants. Ethidium bromide staining of rRNA, 5S RNA, and tRNAs was an indication for equal loading.

When P-sufficient bean plants were subjected to –P treatment for 2 d, *PvHAD1* and miR399 were strongly induced in the root in control plants under a standard 16 h/8 h photoperiod (Figure 6A). Surprisingly, no induced expression of *PvHAD1* and miR399 occurred in continuous dark or in stem-girdled plants. In particular, miR399 expression in leaves was also blocked (Figure 6B). To further modify the C-limiting status of bean plants, we darkened one half of the shoot while exposing the other half of the plants to light during this 2-d period of –P treatment. With regard to light conditions, we designate this process as a 'split-shoot' experiment. miR399 was preferentially induced in leaves exposed to light versus darkened leaves of the same plant (Figure 7A). Furthermore, miR399 induction in the root was strikingly attenuated compared to the control (Figure 7B).

Characterization of *PvHAD1* Promoter in Transgenic Roots

PvHAD1 expression is representative of a local response by Pi sensing in P-deficient roots. The 5′-regulatory region in *PvHAD1* gene is of particular interest for further studies on the mechanism of such a response. Therefore, we isolated the *PvHAD1* gene and sequenced the promoter region 2 kb upstream of the translation start. Two 8-bp sequences matching the consensus for P1BS (5′-GNATATNC-3′) sites were present at –388 and –473 bp upstream of ATG. To assess the activity and fidelity of the *PvHAD1* promoter, we constructed three *PvHAD1* promoter:GUS reporter genes and transformed *Medicago truncatula* roots with *Agrobacterium rhizogenes* containing these constructs. Previous studies of several promoters for genes from P-deficient white lupin have shown to be expressed correctly both spatially and temporally in transgenic *M. truncatula* roots and alfalfa (Liu et al., 2005; Zinn et al., 2009). Each transformed root is an independent event in the *A. rhizogenes* system. In P-deficient *M. truncatula* having transgenic hairy roots containing the *PvHAD1* promoter:GUS reporter, GUS staining was rapid (within 1 h) and intense. When both P1BS sites were mutated or deleted in promoter:GUS fusion, reporter gene staining remained intense in the transgenic hairy roots of *M. truncatula* (Figure 8). In addition, the spatial patterns of all three GUS reporter genes remained the same in meristems, emerging lateral roots, and also in root hairs. For each construct, root segments were excised from at least six composite plants to represent independent transformation events.

DISCUSSION

The molecular mechanism of plant response to Pi fluctuation appears to be highly conserved. The expression of both HAD family phosphatases and miR399 is modulated by Pi availability in various plant species (Baldwin et al., 2001; Misson et al., 2005; Tian et al., 2007; Lin et al., 2008; Valdés-López et al., 2008). Systemic regulation of HAD phosphatase genes by shoot P status has been implicated in both tomato and common bean (Baldwin et al., 2001; Tian et al., 2007; Baldwin et al., 2008). In this report, by assessing the expression patterns of both a phosphatase gene *PvHAD1* and miR399 in common bean, we provide support for the existence of a dual regulatory pathway in common bean, namely systemic signaling of P-deficiency status between shoot and root as well as local sensing of Pi in the root. Furthermore, we demonstrate that C-limitation affects Pi-signaling in Pi-sufficient plants as they are subjected to Pi-withdrawal. We also provide evidence for cross-talk between photosynthate (sugar), P-deficiency gene induction, and miR399.

With regard to local sensing of Pi, we have gained further understanding in two aspects. (1) In addition to the sensing of low Pi as shown in *Arabidopsis* mutants, local Pi sensing also occurs at high Pi concentration to regulate gene expression in P-deficient roots. Such a regulatory mechanism appears to be independent of shoot P status, as marked by steady miR399 transcript levels in the shoot. (2) We demonstrate that systemic signaling of P-deficiency involves cross-talk between C-limitation and P-deficiency by establishing a direct relation between photosynthate (sugar) supply and miR399 induction, namely photosynthate acts upstream of miR399 induction upon Pi withdrawal for the onset of P-deficiency. We have also demonstrated that photosynthetic carbon plays distinct roles in both the onset (+P 14-day-old plants switched to –P for 48 h) and the advanced stage (3-week-old plants) of P-deficiency response in that miR399 induction at the onset of P-deficiency was blocked by restricted photosynthate transport, as evidenced in dark-treated or stem-girdled plants, and miR399 expression at an advanced stage of P-deficiency was not markedly affected by dark treatment or stem-girdling.

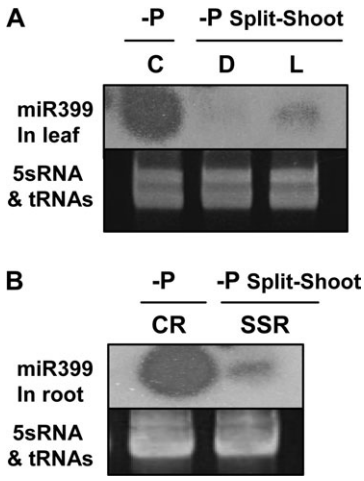

Figure 7. Expression of miR399 in Plants Grown under +P for 14 Days and then –P for 2 Days.

Plants were grown under a normal photoperiod (16 h light/8 h dark). At day 14, half of the leaves were covered to exclude light (D), while the other half of the leaves remained in normal photoperiod (L).

(A) miR399 transcripts were detectable in leaves grown under normal photoperiod (L) but much reduced as compared to the control (C), while darkened leaves had low to non-detectable miR399 transcripts.

(B) miR399 in the roots of split-shoot plants (SSR) was strikingly attenuated as compared to control roots (CR). Ethidium bromide staining of rRNA, 5S RNA and tRNAs was an indication for equal loading.

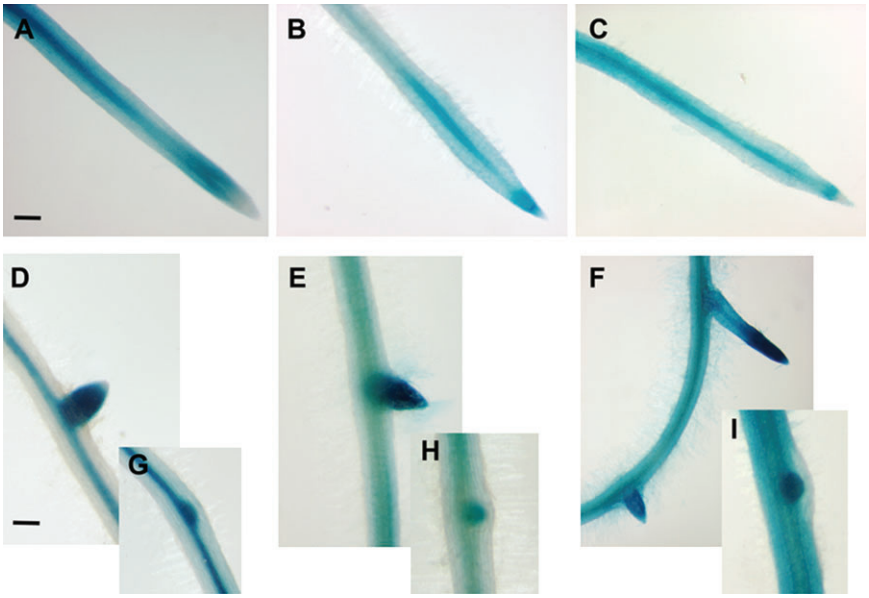

Figure 8. Analyses of *PvHAD1* Promoter Activity in Transgenic Roots of *M. truncatula*.

Histochemical localization of GUS expression in transgenic hairy roots of *M.truncatula*. GUS activity from all constructs was detected in emerging lateral roots and meristems. For 2×P1BS/promoter:GUS construct, staining of the roots was shown in **(A)** elongating roots, **(D)** emerging lateral roots, and **(G)** meristems. Similar patterns were found in transgenic roots with constructs in which both P1BS sites were mutated **(B)** elongating roots, **(E)** emerging lateral roots, **(H)** meristems, or both P1BS sites were deleted **(C)** elongating roots, **(F)** emerging lateral roots, **(I)** meristems. Photos were taken 1 h after GUS staining.

Rapid Down-Regulation of *PvHAD1* Is Due to Local Sensing of Pi in P-Deficient Roots

Down-regulation of *PvHAD1* by Pi occurred within 1 h of Pi and Phi supply to the root (Figures 2C and 5A). The timing of this initial response to Pi provides support for a rapid and localized process for Pi sensing, namely Pi acts as a signal molecule and not merely as a macronutrient. Criteria for a localized signaling process are based upon the rapidity, specificity, and independence of further downstream events. The use of the Pi analog, Phi, meets the above-mentioned criteria, because Phi is perceived by plants as Pi due to structural similarity, but is not metabolized further downstream as a nutrient (Carswell et al., 1996). Therefore, rapid down-regulation of *PvHAD1* by Phi in split-root experiments serves as direct evidence of local sensing of Pi. *PvHAD1* expression was much lower in half of the roots exposed to either Pi or Phi (+Pi half or +Phi half) than in the –P half (Figure 4).

Local sensing of Pi has been observed in *Arabidopsis* mutants. Ticconi et al. (2004) identified the *pdr2* mutant, which cannot sense external Pi of up to 0.1 mM. *pdr2* mutant has a conditional short-root phenotype. Under higher concentration of external Pi or Phi, there is no difference in root architecture between *pdr2* mutants and wild-type plants. In addition, there is enhanced expression of P-responsive genes in *pdr2* mutant under low-Pi conditions. Interestingly, another *Arabidopsis* mutant (*LPR1*) also has a conditional short-root phenotype. *LPR1* phenotype is the result of loss-of-function in a multi-copper oxidase gene. *LPR1* phenotype is noticeable only when the root is in contact with external Pi lower than 5 μM (Svistoonoff et al., 2007). We noted that the phenotypes of these short-root mutants are conditional to a low-Pi concentration, and at a higher concentration of Pi, the phenotype is lost. Recently, Ticconi et al. (2009) showed that *pdr2* encodes a P5-type ATPase, which functions in the endoplasmic reticulum (ER) and is required for the proper expression of SCARECROW (SCR) during Pi deprivation. The underpinning mechanism of rapid down-regulation of *PvHAD*1 by Pi and Phi in P-deficient roots appears to be distinct from that of the above-mentioned *Arabidopsis* mutants. Especially, *PvHAD1* expression in P-deficient roots responded to Pi/Phi concentration of 1 mM within 1 h. Such a dramatic change in *PvHAD1* expression preceded any noticeable reduction in miR399 level in either the root or the shoot (Figure 5B). Since miR399 expression in the shoot is thought to be an indicator of the shoot P status and is crucial for Pi homeostasis under P-deficient conditions (Lin et al., 2008), it is possible that *PvHAD1* response to Pi/Phi in P-deficient roots occurred prior to the change in P status in the shoot. A transition from P-deficiency to P-sufficiency status in the shoot would be marked by miR399 reduction in both the shoot and the root. On the other hand, it should be cautioned that despite the stable level of mature miR399 detected after 1 h of Pi supply to the roots, we do not exclude the possibility that the primary transcript of miR399 could have been reduced prior to the reduction of mature miR399. In *Arabidopsis* seedlings grown in sterile liquid culture under –P conditions, the primary transcript of miR399 is reduced after re-supply of Pi for 30 min or 3 h (Bari et al., 2006).

Carbon Assimilation and Partitioning Are a Checkpoint for the Onset of P-Deficiency

Photosynthetic C assimilation is of central importance for biomass production. The concept of a 'source-sink' C-partitioning between the shoot and the root has been the basis for improving the yield of underground organs of crops. Subsequent partitioning of photosynthate, mostly in the form of sugars (mainly sucrose), between the shoot and the root is achieved via phloem transport. Although sugar transport in phloem serves as a primary pathway for C allocation, sugars transported in the phloem also participate in signaling processes (Chiou and Bush, 1998). Furthermore, C partitioning is tightly coupled to P-deficiency, because a higher root-to-shoot ratio is the hallmark of P-deficiency in most plant species (Hammond and White, 2008; also note the review article by Rouached et al., 2010, Mol. Plant 3: this issue).

Induced expression of miR399 is an indication of the onset of P-deficiency. MiR399 is specifically and rapidly induced in *Arabidopsis* upon Pi withdrawal (Fujii et al., 2005; Chiou et al., 2006; Bari et al., 2006). From our studies with common bean, we also observed that miR399 was strongly induced 2 d after Pi withdrawal from Pi-sufficient plants. While these results corroborate that miR399 is induced by Pi withdrawal, further comparison of miR399 expression patterns between either dark-treated or stem-girdled plants and that of control plants showed a very direct relationship between photosynthate (sugar), miR399 induction, and Pi withdrawal. Despite Pi withdrawal for 2 d, miR399 was not induced in either roots or shoots if plants were in continuous dark. In addition, miR399 was not induced in the roots of stem-girdled plants grown under normal photoperiod (Figure 6B). These results indicate that under C-limitation in either the shoot or the root, Pi withdrawal is not sufficient for the induction of miR399. Both photosynthetic C assimilation and subsequent partitioning to the root are crucial for miR399 induction in roots at the onset of P-deficiency. It is noteworthy that the difference in miR399 induction in roots appears to be an all-or-none difference between control plants and darkened or stem-girdled plants. These findings also suggest that the onset of P-deficiency is very sensitive to photosynthetic C-limitation. C-limitation can occur at varying degrees of severity, just as P-deficiency. To further verify the sensitivity to C-limitation, we carried out 'split-shoot' experiments, namely half of the leaves were darkened and the other half remained in the light during a 2-d period of Pi withdrawal. It is obvious that C-limitation is modulated by the limited leaf area exposed to light. The striking difference between miR399 in darkened leaves versus leaves exposed to light suggests that miR399, as a systemic signal in P-deficiency response, is tightly controlled by the availability of C-source. In addition, horizontal movement of mature miR399 from the light-treated half to the darkened half of the shoot appears restricted, presumably by the

boundaries of the vasculature. With regard to miR399 induction in the root, the difference between control plants and 'split-shoot' plants is significant in that photosynthate (sugar) supply is reduced but unlikely depleted in the roots of 'split-shoot' plants as compared to a complete blocking of sugar transport in stem-girdled plants, yet miR399 induction was almost completely attenuated.

From the expression studies of miR399 and *PvHAD1* during the onset of P-deficiency, we have revealed a mechanism of cross-talk between the two potential signal molecules, photosynthate and miR399, in the systemic signaling of P-deficiency from shoot to root. Our physiological modification of C-availability through leaf shading and stem girdling implicates photosynthate (sugar) as acting upstream of miR399 in P-signaling.

METHODS

Plant Materials and Growth Conditions

Common bean seeds were surface-sterilized with 50% bleach and rinsed with sterile water five times. After 2 d imbibition, seeds were sowed in potted quartz sand and watered once a day with either +P or –P nutrient solution. The +P solution contained 1 mM $Ca(H_2PO_4)_2$ and the –P solution contained no phosphate. For 'split-root' experiments, 3-week-old P-deficient plants were gently harvested and rinsed quickly. Roots were then separated into two containers filled with either +P or –P solution. Plants were left for 1 h under standard light condition in the growth chamber. To study the effect of Pi analog, Pi was replaced with the same concentration of phosphonate (Phi) for 1 h in the split-root system. Plants under various light/dark conditions were handled as previously described (Liu et al., 2005). Nitrate concentration was reduced to 0.1 mM for the nitrate-limiting condition, and no nitrate was supplied for the nitrate-depleting condition for 3 weeks after emergence of seedlings from sand.

RNA Blot Analyses

RNA blot analyses were conducted to study the change of gene expression patterns under various conditions. Total RNA was isolated from root or leaf tissue with the RNeasy KIT (Qiagen, Valencia, CA, USA), separated on formaldehyde-denaturing agarose gels, and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA). A fragment corresponding to the full-length *PvHAD1* coding region was random-labeled and used for hybridization as described in the Zeta membrane manufacturer's instruction.

miRNA Blot Analyses

Total RNA was isolated with TRIzol reagent (Invitrogen) as described by Yoo et al. (2004). Twenty micrograms of total RNA were resolved on denaturing 15% polyacrylamide gels (with 8 M urea) in 0.5 TBE buffer. Gels were stained briefly and photographed for indication of equal loading, then transferred to Hybond N^+ Nylon membrane per the manufacturer's instructions for semi-dry transfer system (Bio-Rad). miRNA was fixed to membrane by UV cross-linking and blot was then hybridized to end-labeled oligo-399 (5′-CCGGGCAAATCTCCTTTGGCA-3′). The sequence of oligo-399 is reverse complementary to that of miR399.

Expression of Recombinant Protein and *In Vitro* Phosphatase Activity Assay

A PCR fragment corresponding to the full-length coding region was amplified with the primers PvAP-EX1 (forward): 5′-CGTGGATCCATGTCTGAAATTGTGGTTGTT-3′; PvAP-EX2: (reverse) 5′-CGGGAATTCGAAATTATGGTCTAACTGGG-3′ and cloned into *Bam*HI/*Eco*RI digested pGEX-2T vector (Pharmacia). The GST:PvAP fusion protein was induced with 0.1 mM IPTG and purified with Glutathione-Sepharose 4B beads as described (Pharmacia). To overcome the low efficiency of elution, the reduced glutathione concentration was doubled in the elution buffer. Both purified GST:PvHAD1 fusion protein and free GST as control were tested for Ser/Thr phosphatase activity with phosphopeptide as described in the instructions of the Serine/Threonine Phosphatase Assay System (Promega).

Construction and Screening of Genomic Libraries

Two genomic libraries were constructed from genomic DNA digested with either *Eco*RI or *Bam*HI/*Sau*3A. Genomic DNA was isolated from young leaves of 1-week old seedlings of common bean (*Phaseolus vulgaris* L.) following a previously described protocol by Junghans and Metzlaff (1990). One hundred micrograms of genomic DNA was digested, fractionated on a 0.6% (w/v) agarose gel, and the DNA within the cloning range (9~23 kb) was cut from gel and purified twice with the GenClean II Kit per the manufacturer's instructions (Bio 101, Carlsbad, CA, USA). The purified genomic DNA was further concentrated and ligated with *Eco*RI or *Bam*HI-digested lambda DASH II vectors, respectively (Stratagene). The amplified libraries were plated out and screened with random-labeled *PvHAD1* cDNA insert. After three rounds of hybridization, single plaques were identified and amplified for high-titer phage stock for extraction of lambda DNA. The insert was cut off with *Not*I and subcloned into the pBluescript KS vector for sequencing.

Construction of Promoter:GUS Reporter Genes and Plant Transformation

The 5′-regulatory region of 2 kb was amplified by PCR with KOD polymerase (Novagen). *Sal*I and *Bam*HI were introduced in the primers PvAP_d1: (forward)5′ggcgtcgacgaccct tcacttgttaaggct-3′; PvAP_d0: (reverse) 5′-ggaggatccaccacaatttcagacatgtttg-3′ for cloning into the T-DNA vector pBI102.2 (Clontech, Palo Alto, CA, USA). For the mutation of both putative P1BS (PHR1 binding sites) in the promoter, two essential base pairs AT in the core of P1BS were changed into CC, namely 5′-GA**AT**ATTC-3′ became 5′-GA**CC**ATTC-3′. Mutations were introduced in both P1BS sites by fusing two overlapping PCR

fragments, which were amplified with primer pairs to incorporate mutations in the first P1BS1 and the second P1BS, respectively. For the first P1BS mutation: (forward) 5′-CCCTAATCGA**CC**ATTCCCATAT-3′ and (reverse) 5′-ATATGG-GAAT**GG**TCGATTAGGG-3′. The conserved sequence for P1BS is underlined with mutated base pairs in bold, namely AT was substituted by CC in forward primer and TA was substituted by GG in reverse primer. For the second P1BS mutation: (forward) 5′-CTAT ACCCAGA**CC**ATTCTGATGGGT-3′ and (reverse) 5′-CAACCCATCAGAAT**GG**TCTGGGTAT-3′. Deletion of the region containing both P1BS sites was carried out by fusing two overlapping PCR fragments, which were amplified with primer pairs based on the 'domain-swapping' strategy. The following two overlapping primers are both flanking the deleted region in order to bridge the PCR fragments by another round PCR amplification: (forward) 5′-GTTAAATGTTTGTGGAAGGGAAGGGCTCAATCTCCAGACCCT CTG-3′; (reverse) 5′-AATCAGAGGGTCTGGAGATTGAGCCCTT CCCTTCCACAAACATTT-3′. Recombinant plasmids were electroporated into *Agrobacterium rhizogenes* (ArQual). Transformation of *Medicago truncatula* and histochemical analyses were performed as described, except that the staining time was for 1 h unless stated otherwise (Ivashuta et al., 2005; Liu et al., 2005).

Accession Numbers

Sequence data from this article can be found in the EMBO/GenBank data libraries under the accession number GU345803.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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